BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

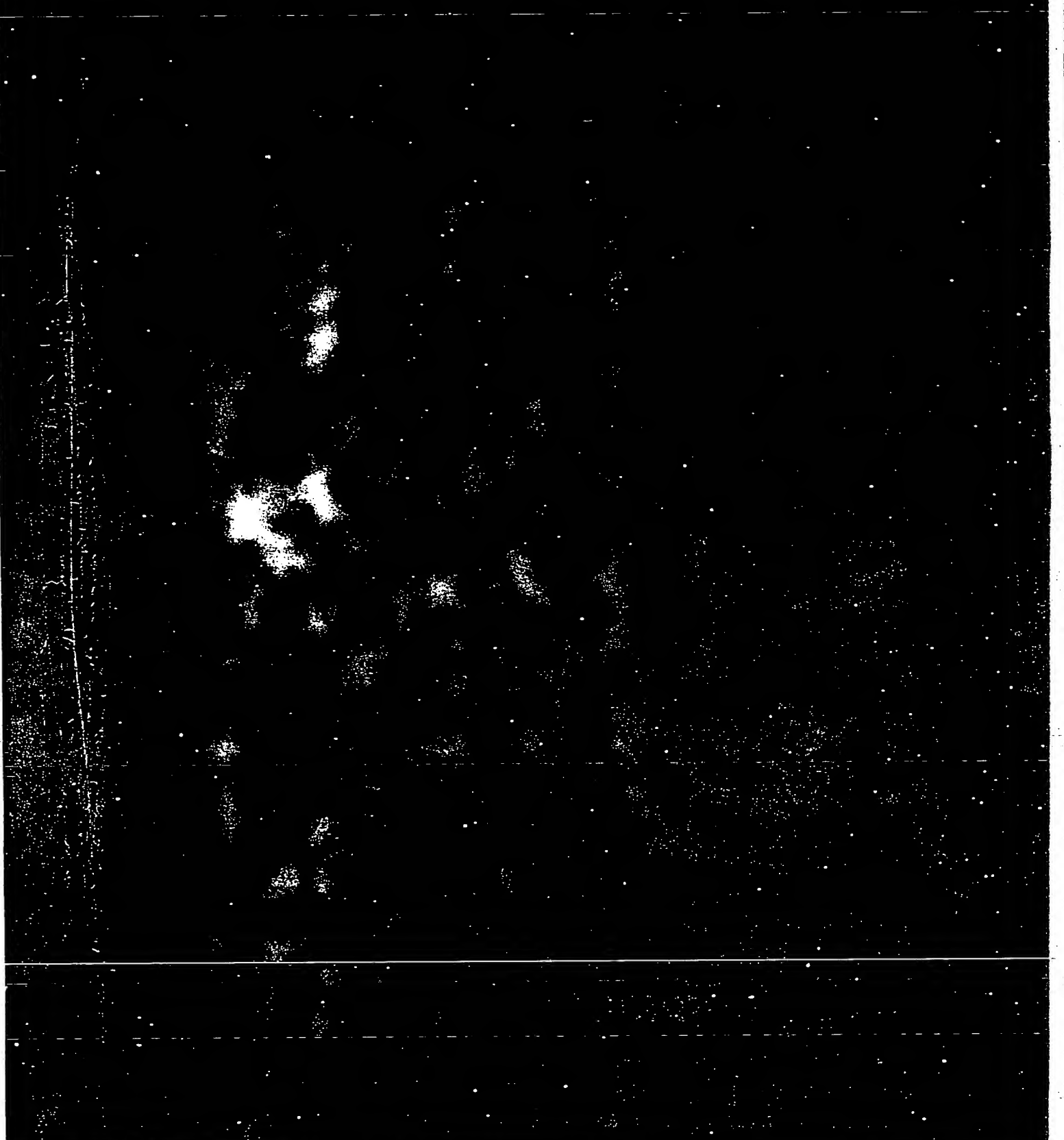
Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

Cell



Volume 75 Number 4 November 19, 1993

Cell

(continued)

The c v r shows a Drosophila fat body, stained for d rsal-related immunity fact r (Dif), which is localized in th nucleus after injury. For details see the article by Ip et al. in this issue.

Cell, Vol. 75, 653–660, November 19, 1993.

Induction of Apoptosis in Fibroblasts by IL-1β-Converting Enzyme, a Mammalian Homolog of the C. elegans Cell Death Gene *ced-3*

Masayuki Miura,* H ng Zhu,* Rocco Rotello,* Erika A. Hartwieg,† and Junying Yuan*
*Cardiovascular Research Center
Massachusetts General Hospital
Charlestown, Massachusetts 02129
†Howard Hughes Medical Institute
Department of Biology
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

Summary

The mammalian interleukin-1β-converting enzyme (ICE) has sequence similarity to the C. elegans cell death gene *ced-3*. We show here that overexpression of the murine ICE (mICE) gene or of the C. elegans *ced-3* gene causes Rat-1 cells to undergo programmed cell death. Point mutations in a region homologous between mICE and CED-3 eliminate the ability of mICE and *ced-3* to cause cell death. The cell death caused by mICE can be suppressed by overexpression of the *crmA* gene, a specific inhibitor of ICE, as well as by *bcl-2*, a mammalian oncogene that can act to prevent programmed cell death. Our results suggest that ICE may function during mammalian development to cause programmed cell death.

Introduction

Programmed cell death (or apoptosis) is a process through which organisms get rid of unwanted cells. Programmed cell death can be considered as a specific type of terminal cell differentiation. In the nematode Caenorhabditis elegans, a genetic pathway of programmed cell death has been identified (Ellis et al., 1991). Two genes, *ced-3* and *ced-4*, are essential for cells to undergo programmed cell death in C. elegans (Ellis and Horvitz, 1986). Recessive mutations that eliminate the function of these two genes prevent normal programmed cell death during the development of C. elegans. Genetic mosaic analysis indicates that *ced-3* and *ced-4* most likely function within cells that die and thus may encode a cytotoxic protein(s) or a protein(s) that controls the activity of a cytotoxic protein (Yuan and Horvitz, 1990). In *ced-3; ced-9* or *ced-4; ced-9* double mutant animals, mutations in *ced-3* and *ced-4* block the lethality of recessive loss-of-function mutations in *ced-9*, and thus *ced-3* and *ced-4* act downstream of *ced-9*, which functions as a cell death suppressor gene (Hengartner et al., 1992).

Understanding the mechanisms of programmed cell death is very important, not only because of the occurrenc of programmed cell death during vertebrate development and tissue homeostasis, but also because abnormally controlled programmed cell death may be an underlying cause of many diseases. In C. legans, the function of *ced-9* can be partially substituted by the mammalian proto-oncogene *bcl-2* (Vaux t al., 1992), suggesting that vertebrate animals may have a similar pathway of programmed cell death. In vertebrates, *bcl-2* functions as a cell death suppressor gene (Vaux et al., 1988; Nuñez et al., 1990; Strasser et al., 1991; Sentman et al., 1991). When overexpressed in hematopoietic cells, *bcl-2* can protect certain cell lines from cell death induced by interleukin-3 (IL-3) deprivation (Vaux et al., 1988; Nuñez et al., 1990). In transgenic animals, *bcl-2* can protect immature thymocytes from a variety of insults, including radiation, glucocorticoid, and anti–T cell receptor antibody-induced cell death (Strasser et al., 1991; Sentman et al., 1991). Additional members of the *bcl-2* gene family, *bcl-x* and *Bax*, have been isolated that may act synergistically or antagonistically with *bcl-2* (Boise et al., 1993; Oltvai et al., 1993).

One of the most direct ways to identify the vertebrate genetic pathway of programmed cell death is to isolate and study the genes homologous to *ced-3* and *ced-4* and to analyze the function of homologs in vertebrate cells. Recently, *ced-4* (Yuan and Horvitz, 1992) and *ced-3* (Yuan et al., 1993 [this issue of *Cell*]) genes have been cloned, and these studies make it possible to identify the cell death genes in vertebrate. The 549 amino acid sequence of the CED-4 protein, deduced from cDNA and genomic clones, contains two regions that are similar to the calcium-binding domain known as the EF hand (Kretsinger, 1987), suggesting that *ced-4* activity and, hence, programmed cell death in C. elegans may be regulated by calcium. However, attempts in cloning the *ced-4* homolog in vertebrates nave been unsuccessful. In contrast with *ced-4*, the amin acid sequence of the CED-3 protein is similar to the mammalian IL-1β-converting enzyme (ICE) (Yuan et al., 1993). ICE is a cysteine protease that cleaves the 33 kd pro-IL-1β into the 17.5 kd biologically active IL-1β (Cerretti et al., 1992; Thornberry et al., 1992). The overall amino acid identity between CED-3 and ICE is 28%, with a region of 115 amino acids (residues 246–360 of CED-3 and 164–278 of ICE) that shows the highest identity (43%). This region contains a conserved pentapeptide, QACRG (residues 356–360 of CED-3), that contains a cysteine known to be essential for ICE function. The similarity betwe n CED-3 and ICE suggests not only that CED-3 might function as a cysteine protease but also that ICE might act as a vertebrate programmed cell death gene. Here we sh w that overexpression of ICE causes Rat-1 cells to undergo programmed cell death and that death can be inhibited by *bcl-2* and *crmA*, a specific inhibitor of ICE (Ray et al., 1992). Thus, ICE may control programmed cell death during vertebrate development, just as *ced-3* controls programmed cell death during C. elegans development.

R sults

Isolati n of Murin ICE cDNA

We cloned the mouse homolog of human ICE from a mouse thymus cDNA library by low stringency hybridiza-

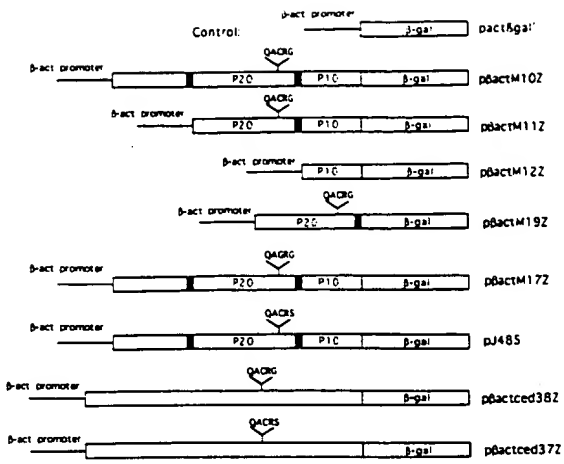


Figure 1. Construction of Expression Cassettes of mICE–*lacZ* and *ced-3–lacZ* Fusion Genes

pβactM10Z contains the intact mICE cDNA fused to the E. coli *lacZ* gene (mICE–*lacZ*). pβactM11Z contains the cDNA coding for the P20 and P10 subunits of mICE fused to the E. coli *lacZ* gene (P20/P10–*lacZ*). pβactM19Z contains the cDNA coding for the P20 subunit of mICE fused to the E. coli *lacZ* gene (P20–*lacZ*). pβactM12Z contains the cDNA coding for the P10 subunit of mICE fused to the E. coli *lacZ* gene (P10–*lacZ*). pβactced38Z contains the cDNA of the C. elegans *ced-3* gene fused to the *lacZ* gene (*ced-3–lacZ*). pJ485 and pβactced37Z contain a glycine to serine mutation at the active domain of pentapeptide QACRG in mICE and CED-3, respectively. pβactM17Z contains a cysteine to glycine mutation at the active domain of pentapeptide QACRG in mICE. pactβgal' is a control plasmid (Maekawa et al., 1991). All plasmids use the chicken β-actin promoter.

tion using human ICE as a probe. This clone, named mICE, is identical to the clone that was isolated by Nett et al. (1992) except for base pair 166, which is an A in our cDNA and resulted in asparagine instead of aspartic acid, as in their clone. This difference may be a DNA polymorphism since our clone was isolated from a thymus cDNA library of mouse B6/CBAFIJ (C57B × CBA) strain while their clone was from a WEHI-3 cell cDNA library. WEHI-3 cells were initially derived from the BALB/c mouse. Subsequent experiments have shown that this DNA polymorphism is in a region not essential for ICE function (see below); thus, it should not have affected our results.

Overexpression of ICE Causes Rat-1 Cells to Undergo Programmed Cell Death

We designed a transient expression system to determine whether overexpression of mICE induces programmed cell death. We fused the mICE cDNA with the Escherichia coli *lacZ* gene and placed the fused gene under the control of the chicken β-actin promoter (pβactM10Z) (Figure 1). The active ICE protein consists of two subunits, P20 and P10, processed fr m a 45 kd precursor peptide (Cerretti t ai., 1992; Thornberry ei ai., 1992). To test the function of th subunits individually, we constructed three additional fusi n g n s, P20/P10–*lacZ* (pβactM11Z), P20–*lacZ* (pβactM19Z), and P10–*lacZ* (pβactM12Z) (Figure 1), which contains the coding regions for th P20/P10, P20, and P10 subunits, r spectively, fused to the E. coli *lacZ* gene.

The mICE–*lacZ*, P20/P10–*lacZ*, P20–*lacZ*, and P10–*lacZ* constructs w r transfected into Rat-1 cells (rat fibroblasts) by calcium–phosphat precipitation. W fix d cells 24 hr after transfection and added X-Gal solution to develop the color reaction (Figure 2). We discovered that most blue (X-Gal-positive) Rat-1 cells transfected with intact mICE–*lacZ* or P20/P10–*lacZ* were round when we stopped color development in 3 hr. When Rat-1 cells were transfected with P20–*lacZ*, P10–*lacZ*, or the control *lacZ* (pactβgal') construct and processed in the same way, most blue cells were flat cells (Figure 2; Table 1). The average round blue cell diameter is 12 ± 1 μm, and the average longitudal size of the flat blue cell diameter is 45 ± 3 μm. This is consistent with the observation made by Jacobson et al. (1993) that the apoptotic cells are about three times smaller in diameter than normal cells. In C. elegans hermaphrodites, most programmed cell death is observed in neuronal lineages (105 of 131 programmed cell deaths; Horvitz et al., 1982). We transfected the mICE–*lacZ* chimeric gene into the neuroblastoma and glioma cell line NG108-15 (Amano et al., 1974), and similar results to those described above were obtained (data not shown). To characterize the nuclear morphology of round blue cells, we stained the mICE–*lacZ*-transfected Rat-1 cells with a rhodamine-coupled anti-β-galactosidase antibody and Hoescht dye. We found that β-galactosidase-positive round cells have condensed and fragmented nuclei (Figures 3A and 3B), a typical feature of cells undergoing programmed cell death (Wyllie, 1981; Oberhammer et al., 1992; Jacobson et al., 1993). Live Rat-1 cells are flat and attach well to the plate, while dying Rat-1 cells are round and eventually detach from the plates. We found that the mICE–*lacZ* chimeric gene-expressing round cell can also be stained by trypan blue (Figures 3C–3E), indicating that X-Gal-positive round cells are dying cells. Thus, our results suggest that overexpression of mICE induces programmed cell death and that the activity depends on both P20 and P10 subunits. If we continued X-Gal color development on Rat-1 cells transfected with mICE–*lacZ* or P20/P10–*lacZ* for 24 hr, additional flat Rat-1 cells turned slightly blue. We interpreted this result as showing that a certain threshold level of mICE is required to induce programmed cell death.

To determine whether cell death caused by overexpression of ICE is specific, we made two mutant ICE fusion proteins: one with a glycine replacing the cysteine in the active site of ICE and the other with a serine replacing the glycine in the pentapeptide active domain of ICE, QACRG (Thornberry et al., 1992), corresponding to the *ced-3* null mutant allele *n2433* (Yuan et al., 1993). We found that both mutations eliminated the ability of mICE to induce Rat-1 cell death (see Figure 2; Table 1). Thus, cell death induced by overexpression of ICE is dependent upon th cysteine protease activity of ICE.

Suppression of Programmed Cell Death by *crmA* and *bcl-2*

The cowpox virus gene *crmA* encodes a 38 kd prot in that can specifically inhibit ICE activity (Ray t al., 1992). If Rat-1 cell death induced by over xpression of mICE is

Figure 2. X-Gal Staining of Rat-1 Cells Expressing Constructs of mICE–*lacZ* and *ced-3–lacZ*

Rat-1 cells were transiently transfected with the constructs shown in Figure 1, fixed 24 hr later, and stained with X-Gal solution for 3 hr. (A and B) pβactM10Z. (C) pβactM11Z. (D) pβactM12Z. (E) pJ485. (F) pβactced38Z. (G) pβactced37Z. (H) pactβgal'. The scale bar in (A) represents 100 μm. The scale bar in (H) represents 50 μm. (B)–(H) are the same magnification.

due to the enzymatic activity of ICE protein, the product of *crmA* should inhibit the cell death induced by the mICE–*lacZ* construct. We transfected the mICE–*lacZ* construct into the Rat-1 cell lines that overexpress *crmA* and developed a X-Gal color reaction as before. We found that we could now obtain a larger percentage of blue cells that showed the flat morphology (Table 1), indicating that the expression of CrmA protein can inhibit ICE-induced cell death. This result suggests that the enzymatic activity of ICE is essential for its ability to kill cells.

In mammals, *bcl-2* pr vents certain cells from und rgoing programmed cell death (Vaux et al., 1988; Nuñez et al., 1990; Strasser et al., 1991; S ntman et al., 1991). Expression of human *bcl-2* in C. elegans has been shown partially to prevent programm d cell death (Vaux et al., 1992); thus, *bcl-2* is functionally similar to the C. elegans *ced-9* gene (Hengartner et al., 1992). Since *ced-9* acts in C. elegans presumably by suppressing the activity of *ced-3* and *ced-4* to protect cells from programmed cell death, we reasoned that if overexpression of ICE causes programmed cell death, such death may be prevented by overexpressing *bcl-2*. When we transfected the mICE–*lacZ* fusion construct into Rat-1 cells overexpressing *bcl-2* and developed the X-Gal color r action as before, w found that a high percentage of blu cells w r now flat, attached cells (Table 1). Thus, the cell death induced by overexpression of mICE can be suppressed by *bcl-2*. This r sult indicates that cell d ath induced by overexpression of mICE is likely to be caused by activation of a normal programm d cell death mechanism.

Table 1. Overexpression of mICE Causes Rat-1 Cells to Undergo Programmed Cell Death

Expression Cassettes	Rat-1	Rat-1/*bcl-2*	Rat-1/*crmA*
pactβgal'	1.4 ± 0.2 (1377)	2.2 ± 0.5 (406)	2.9 ± 0.8 (339)
pβactM10Z	89.8 ± 2.4 (639)	9.9 ± 2.1 (346)	18.8 ± 2.9 (391)
pβactM11Z	93.3 ± 2.7 (570)	13.8 ± 4.3 (350)	24.5 ± 3.4 (538)
pβactM19Z	2.2 ± 0.4 (369)	ND	ND
pβactM12Z	2.4 ± 1.0 (547)	3.3 ± 1.5 (343)	2.6 ± 0.3 (361)
pβactM17Z	2.7 ± 0.8 (389)	ND	ND
pJ485	1.3 ± 0.8 (395)	ND	ND
pβactced38Z	45.0 ± 2.0 (480)	31.5 ± 3.0 (117)	30.8 ± 2.4 (146)
pβactced37Z	3.7 ± 1.6 (345)	ND	ND

The constructs shown in Figure 1 were transiently transfected into Rat-1 cells, Rat-1 cells expressing *bcl-2* (Rat-1/*bcl-2*), or Rat-1 cells expressing *crmA* (Rat-1/*crmA*). Cells were fixed 24 hr after transfection and stained with X-Gal for 3 hr. The data (mean ± SEM) shown are the percentage of round blue cells (cell diameter less than 15 μm) among total number of blue cells counted. The data were collected from at least three independent experiments. Multiple lines that express either *bcl-2* or *crmA* were checked for suppressing mICE-induced cell death, and all showed similar results (data not shown). ND, not determined. The number of total blue cells analyzed is shown in parentheses.

ced-3 Can Cause Rat-1 Cells to Undergo Programmed Cell Death

If mICE is a functional vertebrate homolog of *ced-3*, one possibility is that *ced-3* may also be able to cause Rat-1 cells to undergo programmed cell death. As expected, expression of the *ced-3–lacZ* fusion also induced Rat-1 cell death (see Figure 2; Table 1). If mICE functions in a similar way as *ced-3*, another prediction is that mutations within *ced-3* that eliminate *ced-3* activity in C. elegans should also eliminate its activity in vertebrates. We tested this hypothesis by mutating the glycine residue in the pentapeptide active domain of CED-3, QACRG, to a serine

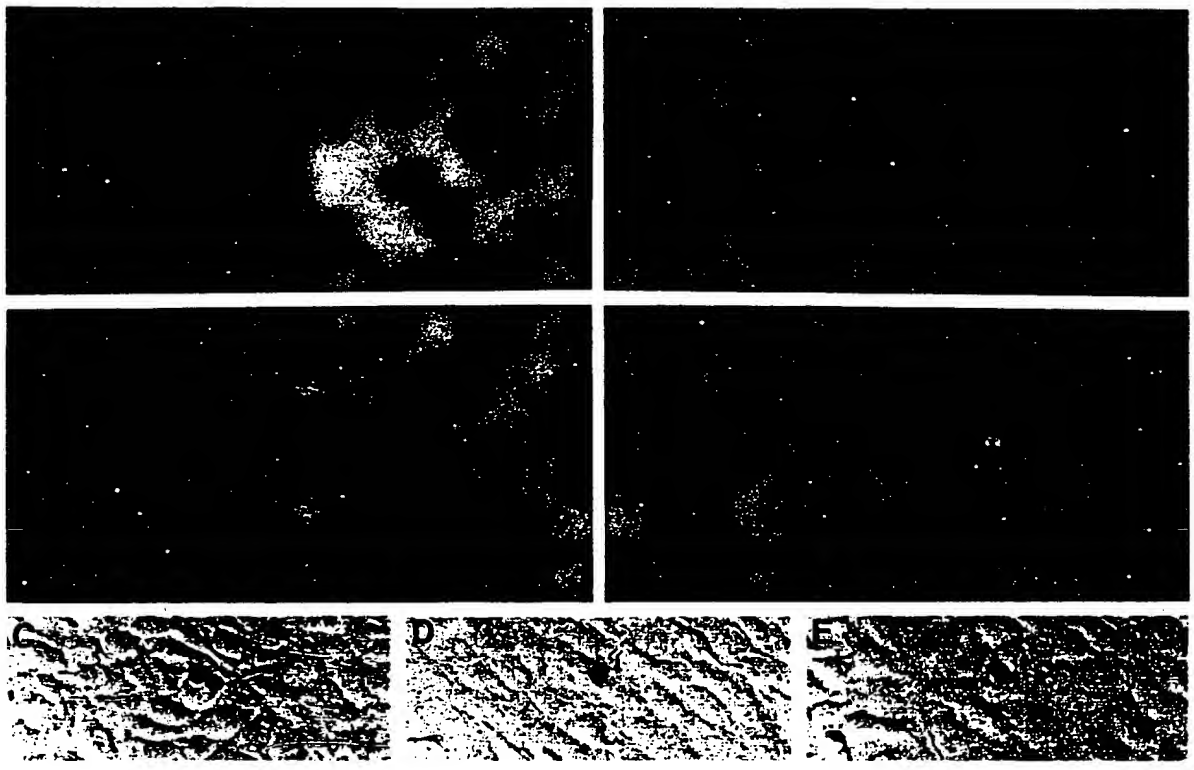


Figure 3. Cells Induced to Die by Overexpression of mICE Have a Condensed Nucleus, a Feature of Programmed Cell Death

(A) Rat-1 cells transiently transfected with control pactβgal' and (24 hr later) fixed and stained by anti-β-galactosidase antibody (top panel) or by Hoescht 33258 dye (bottom panel). The nuclear morphology in β-galactosidase-expressing cells is normal and noncondensed.

(B) Rat-1 cells transiently transfected with pβactM10Z and processed as in (A). The round cell nucleus expressing the mICE–LacZ chimeric protein-expressing cell is condensed and fragmented.

(C) Trypan blue staining of a round dead cell (purple cell).

(D) X-Gal staining of Rat-1 cell transiently transfected pβactM10Z (blue cell).

(E) Trypan blue and X-Gal double staining (blue and purple color in the same cell).

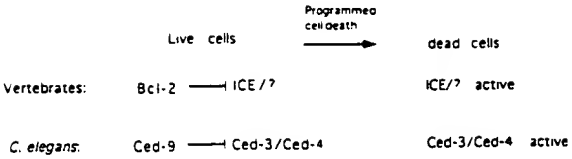


Figure 4. Genetic Pathways of Programmed Cell Death in Vertebrates and in the Nematode C. elegans

In C. elegans, the gene *ced-9* acts to prevent cell death by suppressing *ced-3* and *ced-4*. In cells undergoing programmed cell death, *ced-9* is inactive and *ced-3* and *ced-4* act to cause cell death. In vertebrate animals, *bcl-2* acts to prevent cell death by inhibiting ICE activity. In the cells undergoing programmed cell death, *bcl-2* is inactive and ICE acts together with a putative *ced-4* homolog (shown by a question mark) to cause cell death.

residue, corresponding to the *ced-3* null mutant allele *n2433* (Yuan et al., 1993). We found that this mutation eliminated the ability of *ced-3* to induce Rat-1 cell death (see Figure 2; Table 1).

When we transfected the *ced-3–lacZ* fusion gene into Rat-1 cell lines that overexpress either *crmA* or *bcl-2*, we found that cell death is suppressed, but to a lower extent (Table 1). One explanation of this result is that CrmA and Bcl-2 suppress cell death by directly interacting with cell death proteins and that such interactions with the nematode CED-3 protein are less efficient than with the vertebrate mICE protein.

Discussion

We demonstrate here that overexpression of mICE in Rat-1 cells causes programmed cell death and that mutations in the active domain of ICE eliminate this activity. Our results provide functional evidence that mICE may be a vertebrate cell death gene. Our results suggest that ICE may function to cause programmed cell death during the development of vertebrate animals, similar to CED-3 in C. elegans. Furthermore, Bcl-2 may act normally to suppress the activity of ICE to prevent programmed cell death, as does CED-9 to CED-3 in nematodes. Thus, vertebrate animals may have a genetic pathway of programmed cell death similar to that of C. elegans (Figure 4). We hypothesize that in living cells, Bcl-2 is active, which may directly or indirectly inhibit the activity of ICE; in cells undergoing programmed cell death, Bcl-2 is inactive, and thus ICE is activated, which in turn causes cells to die.

ICE has been identified as a substrate-specific protease that cleaves the 31 kd pro-IL-1β between Asp-116 and Ala-117 to produce the mature 17.5 kd IL-1β. IL-1 is one of primary mediators of the response of the body to microbial invasion, inflammation, immunological reactions, and tissue injury (Dinarello, 1991; diGiovine and Duff, 1990). Although it is not clear what role IL-1β plays in programmed cell death, there is evidence that ICE is activated when certain cells undergo programmed cell death. For example, after murine peritoneal macrophages were stimulated with lipopolysaccharide and induced to undergo programmed cell death by exposure to extracellular ATP, mature active IL-1β was released into the culture; in contrast, when cells were injured by scraping, IL-1β was released exclusively as the inactive precursor peptide (Hogoquist et al., 1991). ICE mRNA has been detected in a variety of tissues, including peripheral blood monocytes, peripheral blood lymphocytes, peripheral blood neutrophils, resting and activated peripheral blood T lymphocytes, placenta, the B lymphoblastoid line CB23, and monocytic leukemia cell line THP1 cells (Cerretti et al., 1992). But only peripheral blood monocytes and THP1 cells have detectable amounts of IL-1β mRNA (Cerretti et al., 1992), suggesting that ICE may have an additional substrate in addition to pro-IL-1β. The substrate that ICE acts upon to cause cell death is presently unknown. One possibility is that it may be a vertebrate homolog of the C. elegans cell death gene *ced-4* (Ellis and Horvitz, 1986; Yuan and Horvitz, 1992). Alternatively, ICE might directly cause cell death by proteolytically cleaving proteins that are essential for cell viability.

The ability of *crmA* to block the cell death induced by overexpression of ICE suggests that one of the in vivo functions of *crmA* may be to block programmed cell death after cowpox virus infection. Latent and/or persistent infections are a part of the lifestyles of many viruses, as the ability to maintain a long-term relationship with their hosts is vital for the survival of viruses. A variety of viral mechanisms have evolved to prolong the life span of host cells. For example, infection of Epstein–Barr virus, a herpes virus responsible for infectious mononucleosis (Henle and Henle, 1979), prolongs the survival of infected B cells. Epstein–Barr virus–coded early lytic cycle protein, BHRF1, a viral homolog of Bcl-2, may be responsible for enhancing B cell survival (Henderson et al., 1993). A baculovirus gene product, p35, has been identified as having the ability to block the host apoptosis response (Clem et al., 1991). Coupling of E1A-induced cell proliferation and E1B-dependent suppression of cell death is required for transformation (Debbas and White, 1993). Programmed cell death may be one of the primary antiviral defense mechanisms of cells. Thus, it is interesting that ICE, the gene responsible for processing active IL-1β, a cytokine produced in response to infection and tissue injury and involved in regulation of the inflammatory and immune responses that contribute to a broad spectrum of host defenses, is also involved in controlling programmed cell death. The close coupling of programmed cell death and IL-1β production may be an efficient way that nature has evolved to counteract viral invasions.

ICE may function in certain cells to cause programmed cell death during vertebrate development, and ICE-induced cell death may be suppressed by Bcl-2. The level of ICE expression is likely to be high in dying cells in our assay since most blue round cells appear within 3 hr of color development. This is similar to *ced-3*: the mRNA level of *ced-3* during the embryonic development of C. elegans, which is the period when most programmed cell deaths occur, is also very high: the level of *ced-3* mRNA is comparable to that of actin 1 (Yuan et al., 1993; J. Y., unpublished data). In addition to the transcriptional regulation of ICE, posttranscriptional regulation is crucial for ICE activity.

ICE itself is proteolytically cleaved from a precursor 45 kd into P20 and P10 subunits for enzymatic activation (C r-retti et al., 1992; Thornberry et al., 1992). We have shown that both P20 and P10 are required for ICE to cause cell death. We hypothesize that part of the activation mechanism of ICE may involve proteolytic cleavage of the 45 kd ICE precursor protein. Consistent with this hypothesis, we detected a processed sized ICE–LacZ fusion protein only in the dying Rat-1 cells transfected with the ICE fusion expression construct, while only the intact fusion protein was detected in transfected living Rat-1 cells (data not shown). A previous study (Thornberry et al., 1992) suggests that ICE cleavage is not autocatalytic. Mechanisms of ICE processing are unclear at present. However, the enzymatic activity of ICE was detected in COS cells transfected with an ICE expression construct, suggesting that overexpression of ICE leads to ICE activation (Cerretti et al., 1992; Thornberry et al., 1992). ICE expression is detected in a wide variety of tissues from the embryonic development stage to adulthood (data not shown). Proteolytic cleavage of ICE activity may be an essential step in ICE activation.

Abnormal regulation of ICE could lead to pathological cell death. High levels of IL-1β have been detected in a number of diseases, including Alzheimer's disease (Griffin et al., 1989), rheumatoid arthritis (Wood et al., 1983; Fontana et al., 1982), septic shock (Waage et al., 1989), and head injury (McClain et al., 1987). Since cleavage of pro-IL-1β is coupled to release (Hazuda et al., 1988), increased IL-1β release suggests that ICE may be activated in these pathological conditions. We propose that inhibition of ICE may be a way treating these diseases or conditions.

Experimental Procedures

Screening of cDNA Library

Standard techniques of molecular cloning were used as described (Sambrook et al., 1989), unless otherwise indicated. A human ICE cDNA was obtained by polymerase chain reaction (PCR) using the human ICE sequence (Thornberry et al., 1992). This cDNA was used as a probe to screen a mouse thymus cDNA library (Stratagene, La Jolla, California). The filters were hybridized in 5× SSPE, 20% formamide, 0.02% Ficoll, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 1% SDS at 40°C for 2 days and washed in 1× SSPE and 0.5% SDS for 20 min, twice at room temperature and twice at 45°C.

Plasmid Construction

pJ415 was constructed by first inserting a 5′ 400 bp BglII–BamHI *crmA* fragment into the BamHI site of the pBabe/puro vector and then inserting the remaining 1 kb BamHI *crmA* fragment into the 3′ BamHI site in a sense direction. pJ436 was constructed by inserting an EcoRI–SalI *bcl-2* fragment into the EcoRI–SalI sites of the pBabe/puro vector (Morgenstern and Land, 1990). To construct fusion genes, the E. coli β-galactosidase gene was taken from the plasmid 407-794.Z (Picard and Yamamoto, 1987) by digestion with BamHI and cloned into pBluescript vector (BSlacZ). Various 5′ deletion fragments of mICE (pJ348) were obtained by PCR. PCR was performed by using synthetic primers (M10 and T3 primer for the whole mICE construct, M11 and T3 primer for the P20/P10 construct, M11 and M13 for the P20 construct, M12 and T3 primer for the P10 construct) and pJ348 as a template. The primer sequences were as follows: M10, AAGTCGACGCCATGGCTGACAAGATCCTGAGGG; M11, AAGTCGACGCCATGAACAAAGAAGATGGCACAT; M12, AAGTCGACGCCATGGGCATTAAGAAGGCCCATA; M13, TTCCCGGGTCATCTTCAAAAATTGCATCCG. The amplified fragments were digested with SalI and SmaI and then cloned into SalI–SmaI sites of BSlacZ. BSced38Z was made by first inserting a SmaI-digested PCR product of *ced-3* cDNA (primers used were M18 and M19; M18, AACCCGGGAGGCCTCCATGATGCGTCAAGATAGAAG; M19, AACCCGGGACGGCAGAGTTTCGTGCTTCCG) into BSlacZ. BSM10Z (mICE–*lacZ* in pBluescript II vector), BSM11Z (P20/P10–*lacZ* in pBluescript II vector), BSM19Z (P20–*lacZ* cloned in pBluescript II vector), BSM12Z (P10–*lacZ* cloned in pBluescript II vector), and BSced38Z (*ced-3*–*lacZ* cloned in pBluescript II vector) were digested with XhoI–NotI, blunt ended by Klenow fragment, and then cloned into pβactSTneoB (Miyawaki et al., 1990) (digested with SalI and blunt ended by Klenow fragment) individually, and the resulted plasmids were named pβactM10Z, pβactM11Z, pβactM12Z, and pβactced38Z, respectively. To mutate the glycine residue to a serine residue in the active domain of mICE, the PCR product of primers m8p/s (ATTCAGGCCTCCAGAGGAGAGAAAC) and mice 8 (GGCACGATTCTCAGCATAGGT), using pJ348 as a template, was digested with SphI and SmaI and then cloned into the SphI–SmaI sites of BSM10Z (pJ483). To mutate the cysteine residue to a glycine residue in the active domain of mICE, the PCR product of M10 and M15 (CAAGGCCTGCCTGAATAATGATCACCTT), using pJ348 as a template, was digested with SalI and StuI, then cloned into BSM10Z that was digested with SphI and blunt ended by T4 DNA polymerase, and then digested with SalI (BSM17Z). To mutate the glycine residue to a serine residue in the active domain of *ced-3*, the PCR products of the C-terminal portion of CED-3 (primers used were M19 and M20; M20, GCAGGCCTGTCGATCGGAACGTCGTGACAATGGATT) and the N-terminal portion of CED-3 (primers used were M18 and M21; M21, ACAGGCCTGCACAAAAACGATTTT) were digested with StuI and SmaI and then cloned into SmaI site of BSlacZ (BSced37Z). pJ483, BSM17Z, and BSced37Z were digested with XhoI and NotI, blunt ended by Klenow fragment, and then cloned into pβactSTneoB individually, and the resulted plasmids were named pJ485, pβactM17Z, and pβactced37Z, respectively.

Cell Culture

Rat-1 cells were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum. The day before transfection, cells were seeded at a density of about 2×10^5 in each of the 6-well dishes. For each well, 1 μg of *lacZ* chimeric construct and 4 μg of calf thymus DNA were coprecipitated with calcium–phosphate according to a standard protocol (Graham and Eb, 1973) and incubated for 3 hr. Cells were washed and incubated for 24 hr. To detect the expression of chimeric gene in transfected Rat-1 cells, cells were fixed with 1% glutaraldehyde for 5 min, rinsed three times with phosphate-buffered saline (PBS), and stained in X-Gal buffer (0.5 mg/ml 5-bromo-4-chloro-3-indoxyl β-galactoside, 3 mM $K_3Fe(CN)_6$, 3 mM $K_4Fe(CN)_6$–$3H_2O$, 1 mM $MgCl_2$, 10 mM KCl, 0.1% Triton X-100 in 0.1 M sodium phosphate buffer [pH 7.5]) at 37°C for 3 hr. To establish Rat-1 cell lines that overexpress *crmA* and *bcl-2*, pJ415 and pJ436 were electroporated into ψCRE retroviral packaging cells (Danos and Mulligan, 1988) using Bio-Rad electroporating apparatus. Supernatant either from overnight transiently transfected ψCRE cells or from stable lines of ψCRE cells expressing either *crmA* or *bcl-2* were used to infect Rat-1 cells overnight in the presence of 8 μg/ml of polybrene. Resistant cells were selected using 3 μg/ml puromycin for approximately 10 days. Resistant colonies were cloned and checked for expression levels using both Northern and Western blots (data not shown).

Antibodies and Immunocytochemistry

Bcl-2 antibodies were from S. J. Korsmeyer and from DAKO (Carpinteria, California). CrmA antisera were made by immunizing rabbits with a E. coli–expressed CrmA fusion protein (pJ434). pJ434 was made by inserting an EcoRI–SalI fragment of *crmA* cDNA into EcoRI–SalI sites of pET21a (Novagen, Madison, Wisconsin), and fusion protein was expressed in an E. coli BL21(DE3) strain. For immunocytochemistry, Rat-1 cells grown on coverslips were prefixed with 4% paraformaldehyde and then postfixed with 5% acetic acid in ethanol. The samples were incubated with anti-β-galactosidase antibody (1:200 dilution, Cappel, Durham, North Carolina) for 1 hr, washed with PBS, incubated with rhodamine-labeled anti-rabbit immunoglobulin G (1:1000 dilution, Cappel) for 30 min, washed in PBS, incubated with Hoescht 33258 dye (final concentration, 5 μM) for 1 min, and washed again in PBS.

Samples were mounted and examined with a Zeiss Axiophot fluorescence microscope. For the trypan blue–X-Gal double staining, cells were washed with PBS and stained with 0.4% trypan blue in PBS for 10 min. Cells were then washed twice with PBS, fixed with 2.5% glutaraldehyde, 2 mM $MgCl_2$, 2 mM EGTA in PBS for 10 min and stained with X-Gal buffer without Triton X-100 for 1 hr.

Acknowledgments

We are in debt to H. R. Horvitz and S. Shaham for stimulating discussions. We thank D. J. Pickup for the *crmA* cDNA clone, S. J. Korsmeyer for the *bcl-2* cDNA clone and the Bcl-2 antibody, H. Wu and R. C. Mulligan for the ψCRE cells, J. P. Morgenstern for the pBabe vectors, K. Yamamoto for the 407-794.Z vector, and T. Furuichi for the pβactST-neoB vector. We thank M. C. Fishman, H. R. Horvitz, and H. Drexler for critical reading of the manuscript. This work is supported in part by grants to J. Y. from Bristol Myer–Squibb, from the National Institute of Aging, and from the Amyotrophic Lateral Sclerosis Association; this work is also supported in part to M. M. by the Mochida Memorial Foundation for Medical and Pharmaceutical Research and a postdoctoral fellowship from the National Institutes of Health.

Received September 20, 1993; revised October 12, 1993.

References

Amano, T., Hamprecht, B., and Kemper, H. (1974). High activity of choline acetyltransferase induced in neuroblastoma × glioma hybrid cells. Exp. Cell Res. *85*, 399–408.

Boise, L. H., González-Garcia, M., Postema, C. E., Ding, L., Lindsten, T., Turka, L. A., Mao, X., Nuñez, G., and Thompson, C. B. (1993). *bcl-x*, a *bcl-2*-related gene that functions as a dominant regulator of apoptotic cell death. Cell *74*, 597–608.

Cerretti, D. P., Kozlosky, C. J., Mosley, B., Nelson, N., Ness, K. V., Greenstreet, T. A., March, C. J., Kronheim, S. R., Druck, T., Cannizzaro, L. A., Huebner, K., and Black, R. A. (1992). Molecular cloning of the interleukin-1β converting enzyme. Science *256*, 97–100.

Clem, R., Fechheimer, M., and Miller, L. K. (1991). Prevention of apoptosis by a baculovirus gene during infection of insect cells. Science *254*, 1388–1390.

Danos, O., and Mulligan, R. C. (1988). Safe and efficient generation of recombinant retroviruses with amphotropic and ecotropic host ranges. Proc. Natl. Acad. Sci. USA *85*, 6460–6464.

Debbas, M., and White, E. (1993). Wild-type p53 mediates apoptosis by E1A, which is inhibited by E1B. Genes Dev. *7*, 546–554.

diGiovine, F. S., and Duff, G. W. (1990). Interleukin 1: the first interleukin. Immunol. Today *11*, 13.

Dinarello, C. A. (1991). Interleukin-1 and interleukin-1 antagonism. Blood *77*, 1627–1652.

Ellis, H. M., and Horvitz, H. R. (1986). Genetic control of programmed cell death in the nematode C. elegans. Cell *44*, 817–829.

Ellis, R. E., Yuan, J., and Horvitz, H. R. (1991). Mechanisms and functions of cell death. Annu. Rev. Cell Biol. *7*, 663–698.

Fontana, A., Hengartner, H., Weber, E., Fehr, K., Grob, P. J., and Cohen, G. (1982). Interleukin 1 activity in the synovial fluid of patients with rheumatoid arthritis. Rheumatol. Int. *2*, 49–53.

Graham, F., and Eb., V. D. (1973). A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology *52*, 456–457.

Griffin, W. S. T., Sranley, L. C., Ling, C., White, L., MacLeod, V., Perrot, L. J., White, C. L., III, and Araoz, C. (1989). Brain interleukin 1 and S-100 immunoreactivity are elevated in Down syndrome and Alzheimer disease. Proc. Natl. Acad. Sci. USA *86*, 7611–7615.

Hazuda, D., Lee, J. C., and Young, P. R. (1988). The kinetics of interleukin 1 secretion from activated monocytes: differences between interleukin-1α and interleukin-1β. J. Biol. Chem. *263*, 8473–8479.

Henderson, S., Huen, D., Rowe, M., Dawson, C., Johnson, G., and Rickinson, A. (1993). Epstein–Barr virus–coded BHRF1 protein, a viral homologue of Bcl-2, protects human B cells from programmed cell death. Proc. Natl. Acad. Sci. USA *90*, 8479–8483.

Hengartner, M., Ellis, R. E., and Horvitz, H. R. (1992). *Caenorhabditis elegans* gene *ced-9* protects cells from programmed cell death. Nature *356*, 494–499.

Henle, G., and Henle, W. (1979). The virus as the etiologic agent of infectious mononucleosis. In The Epstein–Barr Virus, M. A. Epstein and B. G. Achonmg, eds. (Berlin: Springer-Verlag), pp. 297–320.

Hogoquist, K. A., Nett, M. A., Unanue, E. R., and Caplin, D. D. (1991). Interleukin 1 is processed and released during apoptosis. Proc. Natl. Acad. Sci. USA *88*, 8485–8489.

Horvitz, H. R., Ellis, H. M., and Sternberg, P. W. (1982). Programmed cell death in nematode development. Neurosci. Comm. *1*, 56–65.

Jacobson, M. D., Burne, J. F., King, M. P., Miyashita, T., Reed, J. C., and Raff, M. C. (1993). Bcl-2 blocks apoptosis in cells lacking mitochondrial DNA. Nature *361*, 365–369.

Kretsinger, R. H. (1987). Calcium coordination and calmodulin fold: divergent versus convergent evolution. Cold Spring Harbor Symp. Quant. Biol. *52*, 499–510.

Maekawa, T., Matsuda, S., Fujisawa, J., Yoshida, M., and Ishii, S. (1991). Cyclic AMP response element-binding protein, CRE-BP1, mediates the E1A-induced but not the Tax-induced trans-activation. Oncogene *6*, 627–632.

McClain, C. J., Cohen, D., Ott, L., Dinarello, C. A., and Young, B. (1987). Isolation of an interleukin-1-like factor from human joint effusions. J. Lab. Clin. Med. *110*, 48–54.

Miyawaki, A., Furuichi, T., Maeda, N., and Mikoshiba, K. (1990). Expressed cerebellar-type inositol 1,4,5-triphosphate receptor, P_{400}, has calcium release activity in a fibroblast L cell line. Neuron *5*, 11–18.

Morgenstern, J. P., and Land, H. (1990). Advanced mammalian gene transfer: high titer retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. Nucl. Acids Res. *18*, 3587–3596.

Nett, M. A., Cerretti, D. P., Berson, D. R., Seavitt, J., Gilbert, D. J., Jenkins, N. A., Copeland, N. G., Black, R. A., and Chaplin, D. D. (1992). Molecular cloning of the murine IL-1β converting enzyme cDNA. J. Immunol. *149*, 3254–3258.

Nuñez, G., London, L., Hockenbery, D., Alexander, M., Mckearn, J. P., and Korsmeyer, S. J. (1990). Deregulated *bcl-2* gene expression selectively prolongs survival of growth factor-deprived hematopoietic cell lines. J. Immunol. *144*, 3602–3610.

Oberhammer, F. A., Pavelka, M., Sharma, S., Tiefenbacher, R., Purchio, A. F., Bursch, W., and Schulte-Hermann, R. (1992). Induction of apoptosis in cultured hepatocytes and in regressing liver by transforming growth factor β1. Proc. Natl. Acad. Sci. USA *89*, 5408–5412.

Oltvai, Z. N., Milliman, C. L., and Korsmeyer, S. J. (1993). Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programed cell death. Cell *74*, 609–619.

Picard, D., and Yamamoto, K. R. (1987). Two signals mediate hormone-dependent nuclear localization of the glucocorticoid receptor. EMBO J. *6*, 3333–3340.

Ray, C. A., Black, R. A., Kronheim, S. R., Greenstreet, T. A., Sleath, P. R., Salvesen, G. S., and Pickup, D. J. (1992). Viral inhibition of inflammation: cowpox virus encodes an inhibitor of the interleukin-1β converting enzyme. Cell *69*, 597–604.

Sambrook, J., Frisch, E. F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, Second Edition (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).

Sentman, C. L., Shutter, J. R., Hockenbery, D., Kanagawa, O., and Korsmeyer, S. J. (1991). bcl-2 inhibits multiple forms of apoptosis but not negative selection in thymocytes. Cell *67*, 879–888.

Strasser, A., Harris, A. W., and Cory, S. (1991). *bcl-2* transgene inhibits T cell death and perturbs thymic self-censorship. Cell *67*, 889–899.

Thornberry, N. A., Bull, H. G., Calaycay, J. R., Chapman, K. T., Howard, A. D., Kostura, M. J., Miller, D. K., Molineaux, S. M., Weidner, J. R., Aunins, J., Elliston, K. O., Ayala, J. M., Casano, F. J., Chin, J., Ding, G. J.-F., Egger, L. A., Gaffney, E. P., Limjuco, G., Palyha, O. C., Raju, S. M., Rolando, A. M., Salley, J. P., Yamin, T.-T., Lee, T. D., Shively, J. E., MacCross, M., Mumford, R. A., Schmidt, J. A., and Tocci, M. J. (1992). A novel heterodimeric cysteine protease is

required for interleukin-1β processing in monocytes. Nature *356*, 768–774.

Vaux, D. L., Cory, S., and Adams, J. M. (1988). *bcl-2* gene promotes haemopoietic cell survival and cooperates with c-*myc* to immortalise pre-B cells. Nature *335*, 440–442.

Vaux, D. L., Weissman, I. L., and Kim, S. K. (1992). Prevention of programmed cell death in *Caenorhabditis elegans* by human *bcl-2*. Science *258*, 1955–1957.

Waage, A., Brandtzaeg, P., Halstensen, A., Kierulf, P., and Esperik, T. (1989). The complex pattern of cytokines in serum from patients with meningococcal septic shock: association between interleukin 6, interleukin 1, and fatal outcome. J. Exp. Med. *169*, 333–338.

Wood, D. D., Ihrie, E. J., Dinarello, C. A., and Cohen, P. L. (1983). Isolation of an interleukin-1-like factor from human joint effusions. Arthritis Rheum. *26*, 975–983.

Wyllie, A. H. (1981). Cell death: a new classification separating apoptosis from necrosis. In Cell Death in Biology and Pathology, I. D. Brown and R. A. Lockshin, eds. (London: Chapman and Hall), pp. 9–34.

Yuan, J., and Horvitz, H. R. (1990). Genetic mosaic analyses of *ced-3* and *ced-4*, two genes that control programmed cell death in the nematode *C. elegans*. Dev. Biol. *138*, 33–41.

Yuan, J., and Horvitz, H. R. (1992). The *Caenorhabditis elegans* cell death gene *ced-4* encodes a novel protein and is expressed during the period of extensive programmed cell death. Development *116*, 309–320.

Yuan, J., Shaham, S., Ledoux, S., Ellis, H. M., and Horvitz, H. R. (1993). The C. elegans cell death gene *ced-3* encodes a protein similar to mammalian interleukin-1β-converting enzyme. Cell *75*, this issue.